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(54) Title: TEST METHOD

(57) Abstract

A method of determining whether a compound is potentially useful for administration to a mammal in or as antiviral therapy, the method comprising the steps of (i) exposing a responsive system (as herein defined) to the compound and to a stimulating substance (as herein defined), the said system comprising a means for expressing a reporter compound under control of a regulatory region inducible directly or indirectly by the said substance, the said system having a cytokine-response-inhibitory intracellular amount of a viral or cellular protein or portion thereof which inhibits the response to the said substance (generically referred to hereinafter as "the inhibitory protein"), and (ii) determining whether the production or fate of the reporter compound is altered. The cytokine is preferably an interferon (e.g. IFNα) and the inhibitory protein is preferably the amino terminal portion of HBV polymerase. The reporter compound may be cytotoxic such that candidate compounds which cause the cell to die have inhibited the inhibition by the inhibitory protein of the response of the cell to the cytokine.

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TEST METHOD

The present invention relates to a method for testing whether a compound has therapeutic potential as an adjunct to therapy with cytokines.

Cytokines include interleukins and interferons (IFNs). The IFNs are potent anti-viral compounds controlling expression of a large number of genes. Some IFN-inducible proteins have relatively direct antiviral activities, for example 2',5'-oligoadenylate synthetase and dsRNA-dependent protein kinase, whereas the IFN-inducible class I and II HLAs assist in presenting viral antigens to T lymphocytes, facilitating eradication of free virus and recognition and destruction of infected cells. Despite such powerful host defence mechanisms, many viruses still succeed in establishing chronic infections, suggesting that they have evolved ways to avoid the antiviral actions of the IFNs.

More than 200 million people are chronically infected with hepatitis B virus. In such infections, the concentration of circulating αIFN is reduced compared to patients with acute infection, suggesting that one strategem used by HBV to circumvent the effects of IFN may be to inhibit its production (1). Attempts to overcome this deficiency by treating patients with exogenous αIFN have met with considerable success, but many treated patients are not cured.

Following studies into the inhibition of the action and production of IFNs by HBV, we have now devised a test for identifying compounds which are candidates for use as or in antiviral therapy in order to suppress the inhibition by certain viruses of cytokine action and production.

Accordingly, one aspect of the invention provides a method of determining whether a test compound is potentially useful for administration to a mammal in or as antiviral therapy, the method comprising the steps of (i) exposing a responsive system (as herein defined) to the test compound and to a stimulating substance (as herein defined) the said system comprising means for expressing a reporter compound under control of a regulatory region inducible directly or indirectly by the said substance, the said system having a cytokine-response-inhibitory intracellular amount of a substance which inhibits the response to the said stimulating substance (generically referred to hereinafter as "the inhibitory substance") and (ii) determining whether the production or fate of the reporter compound is altered according to whether the test compound is present.

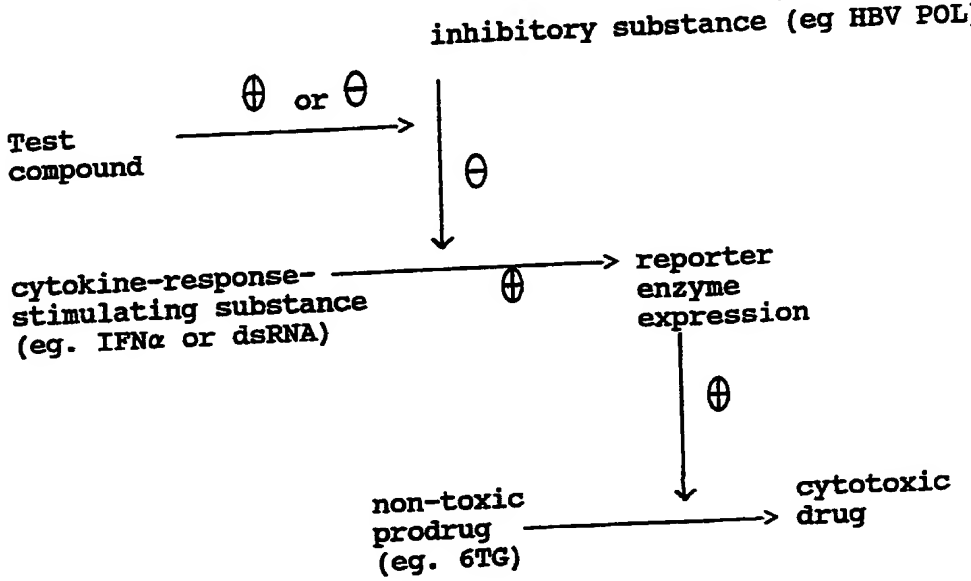
By "responsive system", we mean a system which responds directly or indirectly to the presence of a cytokine or some other agent, for example double-stranded RNA or a virally-encoded protein, to cause cytokine expression or utilisation to increase.

The responsive system is preferably a cell but it may be a cell-free system, for example a crude membrane fraction and a cytoplasmic extract. Most of the following description refers to cells, but cell-free systems, involving means for transcription and translation as necessary, may be used *mutatis mutandis*.

A "stimulating substance" is one which causes cytokine production or utilisation to increase in the type of cell on which the responsive system is based.

In one series of embodiments, the reporter compound is cytotoxic and expression of the compound is indicated by the death of the cells. If the cells die, then the test compound has inhibited the inhibition by the inhibitory substance of the cells' response to the stimulating substance and the compound is considered to be potentially useful. The process is illustrated schematically in Scheme 1:

SCHEME 1



wherein ⊕ indicates enhancement and ⊖ indicates inhibition.

However, the reporter compound may be any compound of which expression may be detected. A specific example is the product of a Class I HLA gene, which in many cells is induced by IFN-α and IFN-γ. The Hep G2 cell line is a well-known example of a cell line which behaves in this way. Thus, in terms of Scheme 1 above, the Hep G2 cells or other cells in which a Class I HLA gene is induced by IFN-α or IFN-γ are transfected with means to express an inhibitory substance, such as any one of the various proteins identified below. Expression of the Class I HLA gene product is detected immunologically, using anti-(gene product) antibodies.

The reporter compound may alternatively be a luciferase,

chloramphenicol acetyltransferase, β-glucuronidase, β-galactosidase, neomycin phosphotransferase or guanine xanthine phosphoribosyltransferase, for all of which genes and detection systems are available. Thus, luciferase-encoding vectors are available from Clontech (Palo Alto, California, USA) and the expression of luciferase may be measured by the method of DeWet *et al* (1987). Similarly, β-galactosidase can be detected using the commercially-available "BluGal" kit (in which a blue colour develops following metabolism by the enzyme of the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) or by optically measuring the metabolism of o-nitrophenol-galactoside at 420 nm.

In the context of using the methods of the invention specifically to detect antiviral compounds useful in combating hepatitis B virus, there may be an advantage in using a hepatic cell line.

A candidate compound identified a useful in the test method of the invention may be antiviral when used alone, since the body's own cytokines may be enough to combat the viral infection, or it may be useful as an adjunct to therapy with another antiviral compound, such as cytokines themselves (especially IFNα), and synthetic drugs such as acyclovir and zidovudine. Relevant viral infections include those involving hepatitis B, HIV, human papillomaviruses, Epstein-Barr virus, delta virus and

hepatitis C.

Preferably, the said stimulating substance is itself a cytokine, such as IFN-α, IFN-β, IFN-γ or IL-6 or is double-stranded RNA or is a virus. In response to the stimulating substance, the system may express or secrete more of a given cytokine or it may express or expose more receptors for a cytokine or may activate or respond in some way to a cytokine or a cytokine-induced factor.

The cytotoxic compound expressed in the cells may be any compound which directly or indirectly kills the cell expressing the cytotoxic compound, or neighbouring cells, in the conditions of use. In a preferred embodiment, the compound is an enzyme, guanine phosphoribosyl transferase, preferably from *E. coli*, which is toxic to HPRT cells when they are maintained in a medium containing 6-thioguanine.

The inhibitory substance will usually be a protein encoded by a virus which infects cells persistently, for example the E1A protein of adenovirus 5 (18), the protein encoded by delta virus (19), the Epstein Barr virus EBNA2 gene product (Ref 32 - see Example 6 below) or the POL protein (or an effective portion or analogue thereof) of hepatitis C (20) or of HIV (24), or viral RNA. Preferably it is HBV polymerase or a portion or analogue thereof (the polymerase or portion or analogue thereof being generically termed "POL" hereinafter).

The POL polypeptide will preferably comprise or consist of the amino terminal domain ("terminal protein", TP) of HBV polymerase or an analogue thereof having conservative substitutions, deletions or additions which do not reduce to an unacceptable level its ability to inhibit cytokine production, secretion or response in the cells concerned. The terminal protein is the amino terminal quarter of the roughly 785 amino acid long multifunctional primary polymerase gene product and can be liberated by protease digestion (12). The most convenient way of ensuring that there is a sufficient intracellular concentration of the inhibitory protein is to transfect the cell line with an inhibition-protein-expressing construct under the control of suitable (non-cytokine inhibited, e.g. constitutive) regulatory regions. Conceivably, though, a sufficient concentration may be arrived at by introduction of the inhibitory protein into the cell in any other way, for example by fusing the cells with inhibitory-protein containing liposomes.

The POL gene encodes a multifunctional protein that includes the terminal protein (TP), a reverse transcriptase and an RNase. Because of the selection based on the IFN-inducible gpt construct, the transferred POL-cells are obliged to produce the HBV reverse transcriptase and the RNase even though these are toxic to the cells. This is the first example of a cell line stably expressing HBV reverse transcriptase. It can therefore be used as the

basis of an assay for HBV reverse transcriptase (HBVRT) inhibiting activity, ie to identify compounds which inhibit HBVRT activity. In order to measure reverse transcriptase activity, one needs both a source of the enzyme and a primer for first-strand DNA synthesis. In the case of HIV, the tRNA primer is available in all cells. However, HBV does not use an endogenous cellular molecule. Rather, it probably uses the terminal protein subdomain of the complex polymerase protein for this purpose. The most likely scenario is: (1) A more than full length RNA is transcribed from viral double-stranded DNA. (2) Terminal protein binds to a specific region of this RNA, near the 3′ end. (3) The first deoxyribonucleoside triphosphate reacts chemically with terminal protein, forming a covalent bond, in a reaction catalysed by HBV reverse transcriptase. (4) The 3′ OH group of this nucleotide then reacts with the next deoxytriphosphate, and so on until the complete RNA strand is copied. (5) The resulting first-strand DNA thus has terminal protein cavalently attached to its 5′ end. (6) Destruction of the RNA template by the RNase H subregion of the viral polymerase and second-strand DNA synthesis then ensue. According to this scenario, it will be necessary to have an appropriate RNA template (with a recognition site for terminal protein) plus the terminal protein and reverse transcriptase subdomains of the complete polymerase protein in order to have activity. If only first-strand DNA synthesis is measured, it may not be necessary to have RNase H. Not all of the necessary

components are likely to be present in the transfected cells defined above since the viral RNA region to which terminal protein binds is missing. It may be that an assay based on fortuitous binding of terminal protein to a cellular RNA is possible, but it would probably be better to supply the viral RNA. A cell-based assay may, for example, demonstrate the synthesis of DNA complementary to the viral RNA. A cell-free assay is another possibility. Here, extracts of the transfected cells described above are supplemented with viral RNA, plus the usual components required for DNA synthesis (triphosphates and the like). Once either type of assay has been established, it is then possible to screen for inhibitors. Reference 25 supplies various details concerning HBV replication, useful in the construction of such assays.

Any convenient cell culture, usually a cell line, may be used in which the construct or constructs described above will function as said, although it is necessary for the cells to be HPRT$^-$ (see ref 17 for details of HPRT$^-$ selection) if the 6TG prodrug detection system is used. Normally, the cell line will be mammalian. Examples include an HPRT$^-$ line of the human cell line HT 1080 and the Hep G2 cell line discussed above.

The interferon-inducible regulatory region may be derived from an IFN-regulated gene such as 9-27 or 2′,5′-oligoadenylate synthetase but is preferably a region

including the promoter-enhancer from the 6-16 gene. We have found that the consensus sequence

GAAATN(N)GAAACT

(where N is any nucleotide and (N) is any or no nucleotide) probably constitutes a necessary although not always sufficient element for response to interferons. Thus, according to the cells, factors E, M and G may be induced by α, β and/or gamma interferons and are bound by interferon-stimulated response elements (ISREs) having the said consensus sequence and forming part of the regulatory region for an interferon-induced gene. Usually, however, it is both more convenient and more effective to use a larger proportion of the regulatory region. In a cell-free system, the inhibition of binding of one of these factors, for example E, M or G, to an ISRE may be monitored by conventional band-shift assays where a ^{32}P-ISRE is used. POL does not inhibit binding of factor E to the ISRE, nor the production of E, but may affect its activation.

Suitable cell lines may be transfected with the said construct or constructs (ie the said "means for expressing") in known ways and may be maintained and used in the test method in ways familiar to those in this art. A particularly preferred cell line which may be transfected with the cytotoxic construct described above and which already contains an IFN-inducible *gpt* gene is 2fTGH

(Reference 4). This may be transfected with the POL-expressing constructs as described below.

The cell line, when used in the assay, needs to be exposed to the stimulating substance. When the stimulating substance is itself a cytokine, it needs to be to extracellular. This is most conveniently provided by simply adding the cytokine to the medium but can be provided by relying on natural secretion of the cell's own cytokine, production and secretion of which may be increased by increasing the copy number or altering the promoter of the relevant gene(s), or by including cytokine-secreting helper cells in the culture. Helper cells may, for example, be obtained by mutation of the cells with a suitable mutagen such as ICR 191 (Polysciences Inc) and selection in HAT medium for cells expressing IFN-regulated genes (specifically, genes for cytokines) in the absence of added IFN. Other cytokine-inducing substances may be provided, extra- or intra-cellularly as appropriate, in convenient ways.

POL has been found to block pathways associated with the production and/or secretion and/or effect of IFNα, IFNβ, IFN-γ and IFN-ω, following response by the cell to such cytokines or to double-stranded RNA. The double-stranded RNA acts as a model for virus infections and as an indication that the response to other viruses will also be blocked.

In the context of the blocking of IFN-γ signalling pathways, whether by POL or any other inhibitory protein, prevention by a candidate compound of such blocking may be detected by monitoring the expression of MHC Class II antigens, or more conveniently, the expression of the prodrug for the cytotoxic compound (e.g. gpt) under the control of a promoter from a MHC Class II antigen gene.

The following discussion relates to the mechanisms underlying the invention and does not detract from the fact that the invention defined above and illustrated in the Examples below defines a method which can be performed by a person skilled in the art to provide a useful result.

So far, we have found that only about 5% of TP-transfected cells are resistant to αIFN and therefore a screening step for such resistance, as described below, is employed to identify useful cell lines. Transfection of the cell lines followed by use of the screening step results in useful cells; in other words, the production of useful cells is not a random or rare event. The rest of the cells have a substantially normal response, as judged by the extent of induction by αIFN of class I HLAs on the cell surface (our unpublished observations using the fluorescence-activated cell sorter). We suspect that only about 5% of the TP transfectants express enough terminal protein to block the response but at present do not have antibody of sufficient quality to test this point directly. The low frequency

with which TP transfectants achieve resistance to αIFN indicates that it may require relatively high expression to substantially affect the IFN response. It is not entirely clear why cells expressing the complete polymerase protein have been found to respond normally to γIFN, in contrast to cells expressing TP. In view of the toxicity of polymerase it is likely that only very small amounts are produced in POL-A$^+$ cells. Perhaps the response to αIFN is more sensitive than the response to γIFN in this situation.

Although the IFNs are potent antiviral agents, many viruses can give rise to chronic infections in humans. Viral inhibition of IFN pathways or suppression of its antiviral effects may allow establishment of these persistent infections. A number of viruses inhibit the production or action of IFN (15) but the role of these effects in disease has not been established. There are strong indications that the E1A proteins of adenoviruses and a protein of Epstein-Barr virus, perhaps EBNA2, can inhibit the response to αIFN in human cells. For the terminal protein of HBV, results obtained by transient cotransfection have correlated well with those from stably transfected cells. We have obtained similar results with the 12S E1A gene product of adenovirus 5, which inhibits the responses to α and γIFNs in transient assays and the response to both IFNs and double-stranded RNA in stably transfected cells.

The TP-expressing cells of the invention have not only lost

their response to αIFN but also fail to respond to γIFN and double-stranded RNA. The effect of HBV DNA in inhibiting responsiveness to double-stranded RNA has been noted before but was ascribed to the core gene. The effect of terminal protein may be specific for IFN-related pathways. It is intriguing to note that there are common elements linking the stimulation of α, β IFN gene expression by double-stranded RNA and the responses of other genes to α and γ IFNs. ISREs governing the response to α IFN can also regulate the response to γIFN and are similar to regulatory elements within the enhancer-promoter regions of the α and β IFN genes themselves. The recently cloned transcription factor IRF-1 (23) is a positive effector for both the β IFN gene and IFN-responsive genes. Double-stranded RNA not only stimulates expression of the α, β IFN genes but also induces IFN-responsive genes directly, in situations where the production of IFN or the response to it is blocked. Terminal protein may inhibit the synthesis or action of a protein required in common for all of these pathways.

We know most about the signalling pathway for α IFN. Terminal protein does not affect either the α IFN receptors or the constitutive level of the Eγ subunit of the key transcription factor E, whose activation almost certainly initiates transcription in response to α IFN. Although we failed to observe activation of the Eα subunit in the cells, the site of inhibition is not known. Expression of the terminal protein region of HBV may inhibit formation of

a precursor of active Eα or may prevent its activation.

Preferred aspects of the invention will now be described by way of example. The references (above and below) are incorporated herein by reference unless stated otherwise.

Figure 1. Response to IFN of cells expressing polymerase or terminal protein. RNase protection assays for mRNAs from POL-A$^-$ or POL-A$^+$ cells (lanes 1-6) or TP-A cells (lanes 7-9) treated with α or γ IFN or untreated. Lanes 10-15, analysis by northern transfer of RNAs from POL-A$^-$ or TP-A cells, probed for an IFN-inducible HLA class I gene or the 1-8 gene family. The assay shown in lanes 1-6 was repeated 3 times and 6-16:actin ratios were quantitated by densitometry.

Figure 2. Band-shift analysis of extracts of TP-A and control cells after treatment with IFN. The POL-A$^-$ cells used as controls are revertants of POL-A$^+$ that no longer express polymerase. Similar results were obtained with control 2fTGH cells. Lanes 1-4, POL-A$^-$ and TP-A cells, untreated or treated with αIFN. Lane 5, mixed extracts from control cells treated with αIFN (lane 2) and untreated TP-A cells (lane 3). Lane 6, U2 cells treated with αIFN, showing no induction of E. Lane 7, mixed extracts from POL-A$^-$ cells treated with γ IFN (containing an induced level of Eγ) and U2 cells treated with αIFN (containing Eα), showing formation of E by complementation. For controls

see lanes 6 and 9. Lane 8, mixed extracts from TP-A cells treated with γ IFN (containing an uninduced level of Eγ) and U2 cells treated with αIFN (containing Eα), showing formation of E by complementation, but at a lower level than in lane 7. For controls see lanes 6 and 10. Lanes 9-10, POL-A⁻ and TP-A cells after treatment with γIFN. For untreated cells, see lanes 1 and 3.

Figure 3. Scatchard analyses of α and γ IFN receptors in TP-A and 2fTGH cells.

Figure 4. RNase protection analyses of cells treated with double-stranded RNA for 4 hr. A. Induction of 6-16 mRNA. B. Induction of βIFN mRNA.

Figure 5. (Example 6). Characterisation of human cells stably expressing Ad5 12S E1A. A. Morphology of parental HT1080 cells (upper panel) and a derivative (HT12S1) expressing the 12S component of E1A (lower panel). B. Immuneprecipitation of 12S E1A polypeptides from HT12S cells labelled with [^{35}S]methionine: comparison with control cells (HT1080) and the polypeptide products of translation of E1A 12S mRNA in a rabbit reticulocyte lysate (cell-free system). Monoclonal antibody M73 is directed against the polypeptide products of both 12S and 13S E1A mRNAs, whereas M2 is specific for the products of the 13S mRNA (Harlow *et al* 1985). C. Comparable levels of α- and γ-IFN receptors on control (HT1080) and E1A expressing

(HT12S1) cells. Scatchard analysis: upper panel α-IFN; lower panel γ-IFN; filled circles, HT1080; open circles, HT12S1.

Figure 6. (Example 6). Inhibition of the response to IFN or double-stranded RNA in cells stably expressing 12S E1A. A. Expression of α- and γ-IFN-inducible 6-16 and 9-27 mRNAs in control (HT1080) and E1A-expressing (HT12S) cells. The time (in hr) of treatment with α- or γ-IFN is indicated above the relevant lanes. B. Expression of β-IFN mRNA in control (HT1080) and E1A-expressing (HT12S) cells in response to treatment with 100 μg/ml poly r(1):r(C) for 3.5 hr. In both A and B assay of the mRNAs was by RNase protection with the protection of γ-actin mRNA serving as an internal control.

Figure 7. (Example 6). IFN-inducible factors in cells stably expressing 12S E1A. A. Inhibition of formation of E and M. Whole cell extracts prepared from HT1080 or HT12S cells with or without treatment for 4 hr with α-IFN were incubated with an end-labelled 39 bp oligodeoxynucleotide probe including the 9-27 ISRE (Methods). DNA-protein complexes were resolved by electrophoresis (Methods). The positions to which complexes corresponding to E and M migrated are indicated to the right. B. The defect in E is in the synthesis or activation of the Eγ subunit. Treatment of HT1080 cells with γ-IFN fails to induce factor E (HTγ, lane 3) - the Eα subunit requires induction by α-

IFN, and thus only the Eγ subunit is present. Mixing of an extract from γ-IFN-treated HT1080 cells with an extract from α-IFN-treated HT12S cells (12Sα, lane 5) results in the formation of E (HTγ + 12Sα, lane 7). This indicates that the Eα subunit is activated in HT12S cells following α-IFN treatment. Treatment of an extract from α-IFN-treated HT1080 cells with NEM (HTα/NEM) inactivates the Eγ subunit and thus E binding is lost (compare lanes 8 and 2). Addition of Eγ subunit by mixing HTα/NEM with an extract from γ-IFN-treated HT1080 cells allows formation of E (lane 10). This is not the case if HTα/NEM is mixed with extract from γ-IFN-treated HT12S (lane 9), indicating that HT12S cell extracts are deficient in the Eγ subunit.

Figure 8. (Example 6). Effect of deletion of conserved regions on the ability of E1A to inhibit the IFN response. A. Transient co-transfection assays in HeLa cells. Co-transfections (molar ratio 1:1) were with the predominantly α-IFN-inducible 6-16 promoter driven CAT construct 203.2.14 (Table IA) and either a control plasmid (lanes 1 to 3) or plasmids expressing a wild-type E1A gene (pCe, lanes 4 to 6) or CR2 (G3/2, lanes 7 to 9) or CR1 (G5/3, lanes 10 to 12) E1A deletion constructs. Transfections and α- or γ-IFN treatment, as indicated, were as described in Table I. The CAT activities represent the average of duplicate plates for one experiment performed three times. B. Expression of α- and γ-IFN-inducible 6-16 and 9-27 mRNAs in control cells (HT1080) and cells stably transfected with either a 12S E1A

construct (HT12S) or an E1A CR1 deletion construct (HTG5/3, Methods). Treatment with IFN was for 6 (α) or 24 (γ) hr. Assay of the RNA was by RNase protection with γ-actin serving as an internal control.

Figure 9. (Example 6). Effect of co-expression of the 12S and 13S products of the wild-type E1A gene on the IFN response. A. RNase protection analysis of IFN-inducible 6-16 and 9-27 mRNAs from control cells (HT1080) and cells stably expressing either 12S and 13S E1A mRNAs (HTCe2) or only the 12S mRNA (HT12S). Treatment with α- or γ-IFNs was for 6 or 24 hr respectively. B. Inhibition of IFN-inducible factors. Whole cell extracts from HT1080 and HTCe2 cells, with or without treatment with α-IFN (4 hr) or γ-IFN (16 hr) as indicated, were incubated with an ISRE-containing probe and analysed as in the legend to Fig 3A. The positions to which the complexes corresponding to the α-IFN-inducible factors E and M, or the γ-IFN-inducible factor G, migrated are indicated on the right.

Abbreviations:

IFN, interferon; HBV, hepatitis B virus; SV40, simian virus 40; CAT, chloramphenicol acetyl transferase; POL, rPOL, TP, POL-COOH, constructs encoding HBV polymerase, polymerase in reverse orientation, the terminal protein domain of polymerase, and the reverse transcriptase plus RNAse H domains, respectively; gpt, guanine phosphoribosyl

transferase; 6TG, 6-thioguanine; ISRE, interferon-stimulated response element; HPRT, hypoxanthine-guanine phosphoribosyl transferase.

MATERIALS AND METHOD

Plasmid constructions. Polymerase constructs were made by excising the open reading frame from a plasmid containing the genome of HBV, subtype ayw (2), and subcloning it into the polylinker adjacent to the SV40 promoter of the vector pJ3omega (5). A polymerase insert was constructed by excising and recircularizing the HBV genome. The 2.5-kb *Bsp*MII-*Hgi*AI fragment was linked to a synthetic oligonucleotide designed to complete the open reading frame and to include a new *Cla*I site adjacent to the ATG for the polymerase gene. The recircularized fragment was cut with *Cla*I and cloned into the *Cla*I site of pJ3omega in the correct (POL) or inverse (rPOL) orientations. The construct TP was made by excising the *Ban*II-*Kpn*I fragment from pJ3POL and recircularizing the plasmid after blunting the ends with T4 polymerase. Enzymes purchased from New England Biolabs were used according to the manufacturer's instructions.

Transient assays. HeLa cells (6×10^5) were grown in 6-well plates and transfected by the calcium phosphate technique (6) with 20 or 40 μg of POL or the same number of molar equivalents of the other constructs, plus 3 μg of a

reporter construct in which 1040 bp of the 6-16 promoter-enhancer region is linked to the chloramphenicol acetyl transferase (CAT) gene (6). The cells in two wells were treated with 500 IU/ml of a highly purified mixture of αIFNs (7), 10^8 IU/mg of protein, provided by Wellcome Research Laboratories, and the cells in 2 wells were treated with 500 IU/ml of γIFN (2 x 10^7 IU/mg, a gift of Dr G R Adolph, Ernst-Boehringer-Institut für Arzneimittel-Forschung, Vienna, Austria). Cytoplasmic CAT activity was measured in duplicate 24 hr later (6).

Stable transfections of 2fTGH cells. Each test plasmid (20 μg), mixed with 2 μg of a plasmid containing either the neomycin or puromycin resistance gene under control of an SV40 promoter, was transfected in duplicate into 4 x 10^5 2fTGH cells (4) using the calcium phosphate technique (6). After 24 hr, one transfected pool was selected in G418 or puromycin and the other was selected in 6-thioguanine (6TG), IFN and test compound. Selection was withdrawn after 10 days and the colonies were counted. For initial transfections with the POL construct, colonies selected in G418 were pooled and then selected again in 6TG plus IFN.

Analysis of IFN-inducible RNAs. Cells were treated with 500 IU/ml of α or γ IFN for 6 or 24 hr, respectively. Reducing the duration of treatment with γIFN reduced the signal in all cases but did not lead to a different relative response. To analyze the response to double-

stranded RNA, cells were treated with 100 μg/ml of poly rI.rC (Pharmacia) for 4 hr in serum-free medium. Cytoplasmic RNA was extracted with phenol/chloroform (8) and annealed overnight to riboprobes labelled with [^{32}P] UTP, prepared from Sp6 plasmids containing a 160-bp probe from the 5′ end of the human 9-27 gene, a 190-bp probe from the first exon of the human 6-16 gene, a 277-bp probe from the human βIFN gene or a 130-bp γ-actin probe (all kindly supplied by Dr S Goodbourn, Imperial Cancer Research Fund). Single-stranded RNA was destroyed with RNases T1 and A and the double-stranded RNAs remaining were separated in a denaturing 7% acrylamide gel (8). Northern transfers were performed as described previously (4).

Cell staining. Cells grown for 24 hr on glass cover slips were fixed in methanol and incubated with primary antibody at a dilution of 1:1000 and then incubated in secondary and tertiary antibodies as described previously (9). Rabbit anti-polymerase serum was from Dr M McGarvey (9), St Mary's Hospital, London.

Band-shift analyses. Whole cell extracts were prepared from cells treated with 500 IU/ml of α or γ IFN for 4 hr (10). Extracts containing 20 μg of protein were incubated with a ^{32}P-labelled 39-bp oligonucleotide probe corresponding to the 9-27 IFN-stimulated response element (ISRE) plus flanking sequences. The probe binds to the IFN-activated transcription factor E with high affinity

(27,28). The complexes were separated in 6% acrylamide gels and analyzed by autoradiography. In some experiments equal volumes of extracts were mixed at room temperature for 15 min before adding the probe.

Analyses of IFN receptors. The assays were performed as described previously (22,29. In brief, cells (2 x 10^6 for γIFN and 4 x 10^5 for αIFN) were incubated with I^{125}-labelled α_2-IFN (reference 11, specific activity 90 μCi/μg) or γIFN (a gift from Dr J Wietzerbin, Marie-Curie Institut, Paris, France, specific activity 180 μCi/μg) for 90 min in the presence or absence of a 1000-fold excess of unlabelled competitor IFN. The cells were washed and detached by pipetting in cold binding buffer and the amount of bound radioactivity was determined. The values were corrected by subtracting uncompeted background.

EXPERIMENTS AND RESULTS

EXAMPLE 1

Transient cotransfection assays. HeLa cells were co-transfected with a reporter construct, in which expression of CAT is regulated by an IFN-inducible promoter, and constructs that express the polymerase protein of HBV under control of a simian virus 40 (SV40) promoter (Table 1). About 40% of the cells transfected with the polymerase construct stained only with the appropriate antibody when

tested after 3 days (data not shown). A construct encoding HBV polymerase (POL) reduced the response to both α and γ IFN substantially, whereas the construct rPOL, in which the polymerase open reading frame is reversed, had no effect. When CAT expression was controlled by the constitutive SV40 promoter, cotransfection with the POL construct was not inhibitory when compared with rPOL (Table 1).

The polymerase gene encodes a multifunctional protein which includes terminal protein (at the amino terminus), a reverse transcriptase and an RNase (at the carboxyl terminus) (12). To determine which of these domains might be responsible for inhibiting the response to IFN, constructs encoding only terminal protein (TP) or both reverse transcriptase and RNase (POL-COOH) were tested. In transient transfection assays, TP inhibited the response to α and γ IFNs profoundly but POL-COOH had little or no effect (Table 1).

Legend to Table I. A. Effect of co-transfection of 12S or 13S E1A-expressing constructs (pJF12 and pJN20) on expression of CAT activity from IFN-inducible constructs in HeLa cells. The inducible constructs were: for the 6-16 gene promoter/enhancer, nucleotides -603 to +454 of the predominantly Type I IFN-inducible 6-16 gene in pSVOCAT (203.2.14, ref 6); for the 9-27 gene promoter/enhancer, nucleotides -213 to -86 of the Type I and II IFN-inducible 9-27 gene in pCATb′ (pPAWCATb′, Reid *et al* 1989) and for

the 6-16 ISRE, GGGAAAATGAAACT 5′ of the tk promoter in pBLCat2 (474.1.1, Reid *et al* 1989). Co-transfection was at a molar ratio of 1:1. Treatment with 1000 IU/ml of either α- or γ-IFNs was for 30 hr starting 16 hr after transfection. Duplicate plates of cells were assayed for CAT activity and the fold inductions shown are the average of at least three experiments. B. Effect of co-transfection of 12S or 13S E1A-expressing plasmids on the constitutive expression of CAT activity from constructs driven by the herpes simplex virus tk promoter (pBLCAT2, ref 33) or the Rous sarcoma virus long terminal repeat (pRSVCAT, ref 34). Co-transfections and CAT assays were performed as for Table IA. Duplicate plates of cells were assayed for CAT activity and the fold inductions shown are the average of at least three experiments.

Table 1. Effects of individual HBV open reading frames on expression of an ISRE-CAT construct in IFN-treated cells

Test construct	Reporter construct*	CAT Activity from a typical experiment (cpm) No IFN	α	gamma	Fold induction with αIFN+		Fold induction with γIFN+	
Vector alone	6-16 CAT	86	5988	538	77	(70-83)	6	(5.5-6.5)
Core	6-16 CAT	114	4532	ND#	50	(40-85)	ND	
Surface	6-16 CAT	381	43126	ND#	80	(57-113)	ND	
X	6-16 CAT	568	67264	ND#	85	(74-118)	ND	
POL	6-16 CAT	49	945	76	23	(19-26)	1.8	(1.6-1.9)
rPOL	6-16 CAT	76	4091	286	55	(50-70)	4	(3.5-6.5)
Vector alone@	6-16 CAT	63	8747	418	100	(70-130)	6	(4.8)
POL@	6-16 CAT	56	1723	158	40	(30-49)	2.6	(2.0-3.2)
TP	6-16 CAT	50	537	75	8	(7-10)	1.5	(1.0-1.7)
POL-COOH	6-16 CAT	81	5249	320	65	(30-88)	4	(3-4.5)
POL	SV40 CAT	33607	ND	ND	ND		ND	
rPOL	SV40 CAT	41640	ND	ND	ND		ND	

Cells were cotransfected with an HBV construct and a reporter construct in the molar ratio 15:1, unless indicated otherwise. *6-16 CAT, IFN-responsive promoter; SV40 CAT, constitutive promoter

+Means (ranges) from three experiments

#ND, not determined

@Ratio of transfection was 7:1

EXAMPLE 2

Analysis of stable cell lines expressing the complete polymerase protein. We (unpublished data) and others (13) have not been able to express HBV polymerase stably in human cells. To overcome this problem we used the cell line 2fTGH, which contains the *Escherichia coli* guanine phosphoribosyl transferase (gpt) gene under control of the IFN-inducible promoter-enhancer of the 6-16 gene (4). In the presence of αIFN, gpt is expressed and the cells die in medium containing 6TG. When the response to αIFN is inhibited, the cells survive in 6TG plus αIFN. Since 2fTGH cells have a very low frequency of spontaneous reversion (4), they can be used to select for agents that inhibit the response to αIFN, in this case the polymerase gene of HBV. However, although expression of polymerase does inhibit the response to IFN, this protein is almost certainly toxic. Consistent with this, (i) only a relatively small number of cells stably transfected with the POL construct survived selection in αIFN plus 6TG (Table 2) and all of these grew slowly; (ii) three out of six clones analyzed further lost both detectable expression of polymerase and inhibition of the IFN response (Table 2) upon removal from selection in an attempt to improve their growth. POL-A^+ cells continued to express detectable polymerase when grown under selective conditions. Expression was seen both in the cytoplasm and nucleus using a previously described antiserum (9). POL-A^+ cells also showed an approximately 3-fold decrease,

compared with polymerase-negative POL-A$^-$ revertant cells, in the levels of 6-16 mRNA induced in response to αIFN (Fig 1, lanes 1-6) or βIFN (data not shown). The response to γIFN, assessed by using the γIFN-inducible gene 9-27 (14), was unimpaired. Northern transfers from POL-A$^+$ and POL-A$^-$ cells were probed for expression of other IFN-inducible mRNAs 1-8: HLA class I, 2′,5′-oligoadenylate synthetase and ISG 54. Again, the response to αIFN was reduced by 2-3 fold in POL-A$^+$ cells but the response to γIFN was normal (data not shown). It is unlikely that the reduction in the response to αIFN is due to general toxicity of polymerase since the levels of mRNAs for the constitutively expressed genes γ-actin (Fig 1) and glyceraldehyde-3-phosphate dehydrogenase (data not shown) were unchanged and the response to γIFN was not impaired. In summary, cells expressing HBV polymerase stably can be obtained by selection for failure to respond to αIFN. They grow very slowly and lose polymerase expression readily when out of selection. When polymerase is expressed, the response to αIFN is decreased significantly.

EXAMPLE 3

Analysis of subdomains of HBV polymerase. Fortunately, more efficient inhibition of the response to IFN is seen without accompanying toxicity in cells stably transfected with a construct capable of expressing the terminal protein domain of HBV polymerase. 2fTGH cells transfected with TP

gave rise to several clones resistant to αIFN plus 6TG (Table 2). When examined by RNase protection clones TP-A (Fig 1, lanes 7-9 and 13-15) and TP-B (data not shown) revealed no response to α or γ IFN. TP-A stained with an anti-polymerase serum (9) and maintained expression of terminal protein even in the absence of selection for 6 months, showing that this protein is not cytotoxic. The two TP clones grew at a normal rate in either selective or non-selective media. Northern transfers from IFN-treated TP-A cells were probed with cDNAs for the IFN-inducible genes HLA class I and II, 2′,5′-oligoadenylate synthetase and 1-8. The normal low constitutive level of expression of these genes was maintained, but none was induced by either α or γ IFN (Fig 1, lanes 7-15, and data not shown). In parallel experiments, transfection with POL-COOH followed by selection for the cotransfected puromycin gene gave 3 colonies while TP or the control plasmid pJ3omega each gave about 100 colonies. This result suggests that the reverse transcriptase plus RNase H region of the polymerase protein may be responsible for the toxicity of the complete protein. When selected in 6TG plus IFN, POL-COOH transfectants gave no colonies (Table 2).

Table 2. Frequency of IFN resistance in stably transfected 2fTGH cells.

Test Construct	Number of clones Transfected	Surviving in 6TG + IFN	Insensitive to αIFN
rPOL	880	1	0/1*
Core	303	1	0/1*
POL	1060	18	3/6+
TP	135	5	2/2
POL-COOH	37	0	-

*Analysis by northern transfer of mRNAs induced in these clones indicated that both responded normally to αIFN (data not shown). Therefore their failure to express gpt was not due to inhibition of the IFN response.

+Six of the original 18 clones were grown for 3 weeks without selection before assaying their response to αIFN. They continued to grow very slowly (doubling time 5 days, parental 2fTGH cells double in 1 day).

EXAMPLE 4

Inhibition of responses to α and γ IFNs. Binding of αIFN to its receptor activates the latent transcription factor

E (or ISGF3) in the cytosol (21,22). (Factors E and ISGF3 are very likely to be the same, even though this point has not been formally proved. We use E for convenience, with this reservation in mind). Active E moves rapidly to the nucleus where it binds to ISREs near the promoters of αIFN-inducible genes. E is not activated in TP-A cells treated with αIFN (Fig 2, lanes 1-4). An extract of TP-A cells did not affect the binding of active E to an ISRE in a band-shift assay (Fig 2, lane 5). Active E contains at least 2 different subunits (22). E-γ is present in untreated cells, but its levels are increased by treatment with γIFN in many cells, including 2fTGH (see below). In intact cells, Eα is activated by αIFN and then combines with E-γ. Cell-free preparations containing only E-γ or only active Eα generate active E factor when combined. The mutant cell line U2, derived from 2fTGH cells, lacks functional E-γ but contains normal amounts of active Eα after treatment with αIFN. Thus, αIFN-treated U2 cells provide a convenient source of active Eα without E-γ (Fig 2, lanes 6 and 7). When extracts of γ IFN-treated TP-A cells were mixed with extracts of αIFN-treated U2 cells, active E was formed (Fig 2, lane 8), showing that TP-A cells contain E-γ but not Eα. Note that more E is formed in the complementation assay with γ IFN-treated POL-A cells (lane 7) than with γ IFN-treated TP-A cells (lane 8), indicating that the increase in E-γ induced by γ IFN is likely to be blocked in TP-A cells. Since TP-A and parental 2fTGH cells have approximately the same numbers of αIFN receptors with about

the same affinities (Fig 3A), terminal protein must block the formation of active Eα at some point distal to the binding of IFN to its receptor.

The transcription factors involved in the primary response to γ IFN have not yet been described. However, treatment with γ IFN is known to induce the secondary synthesis of one or more ISRE-binding factors, called IBP-1, ISGF2 or G. The γ IFN receptor is present in normal numbers and with normal affinity on TP-A cells (Fig 3B) but the band-shift complex containing G is not formed with extracts of TP-A cells treated with γ IFN (Fig 2, lane 10). Terminal protein may interfere either with the primary response to γ IFN or with the secondary formation or function of G.

EXAMPLE 5

Effects of other inducers. The response of TP-A cells to double-stranded RNA was evaluated for the 6-16 and βIFN genes. In both cases, induction was apparent in controls but not in TP-A cells (Fig 4). The 6-16 gene is induced as a primary response to double-stranded RNA as well as secondarily through induction of α, βIFNs. The predominant pathway after only 4 hr of treatment with poly rI.rC is likely to be the direct one. Apparently, neither mechanism of induction is functional in TP cells.

EXAMPLE 6

Summary

Inhibition of the cellular response to interferons by the adenovirus E1A oncogene. Expression of the adenovirus type 5 E1A oncogene inhibits the response of interferon (IFN)-inducible promoter constructs to Type I ($\alpha\beta$) and II (γ) IFNs in transient transfection assays. Human cell lines stably expressing E1A 12S mRNA and protein have been isolated in which the antiviral state and the induction of a number of genes in response to α and γ IFNs is inhibited. A 14 bp promoter proximal Interferon-Stimulable Response Element (ISRE) has previously been defined which mediates gene induction by α and γ IFNs. In the E1A expressing cells there is a substantial reduction in the levels of factors E and M or G which bind to the ISRE in response to α or γ IFNs, respectively. In the case of E, the defect is in the E γ subunit. No evidence was obtained for an effect of E1A on preformed E or on the association of Eα and E-γ subunits. Induction by γ IFN of class II HLA genes, which is thought to involve a different ISRE, and of β-IFN by ds RNA, was also inhibited, suggesting that more than one pathway is affected. The inhibitory activity of E1A was lost upon deletion of the CR1 domain. An essential component(s) of the α and γ IFN response pathways must, therefore, be subject (directly or indirectly) either to the transcriptional repression function of an E1A gene

product or to moldulation of activity by such a product. It should be noted that the E1A protein affects a different subunit of E factor from the subunit affected by hepatitis B terminal protein (E γ or Eα, respectively).

The following describes this Example in more detail.

The replication of adenoviruses in human cells is relatively resistant to IFN treatment, partly due to the expression of the virus-associated (VA) RNAs. These short RNAs bind to the IFN-inducible double-stranded (ds) RNA-dependent protein kinase and prevent its activation. A similar function has recently been ascribed to the EBER RNAs of Epstein-Barr virus (26). An additional mechanism was suggested when it was shown that infection by wild-type adenovirus, but not an E1A-defective mutant, could inhibit the establishment of the antiviral state (27). The induction of certain cellular genes by α-IFN was subsequently shown to be inhibited either by infection with wild-type Ad5 or by expression of E1A in a transient co-transfection assay (18).

At early times of infection and in transformed cells the E1A gene expresses 12S and 13S mRNAs by differential splicing of a single primary transcript. The protein products have identical amino and carboxy termini but differ by 46 internal amino acid residues unique to the larger protein. Comparison of amino acid sequences from

different adenovirus serotypes identified three highly conserved regions. Conserved region (CR) 3, unique to the 13S mRNA products, is required for transactivation of viral early genes by E1A. CR1 and CR2 are involved in cell transformation, induction of cellular DNA synthesis and transcriptional repression of viral and cellular genes.

Here, human cell lines stably expressing the 12S or 12S plus 13S E1A products are used for the detailed examination of the effects of such expression on gene induction in response to α- and γ-IFNs and double-stranded RNA (dsRNA).

MATERIALS & METHODS

Growth of cells, transfections, CAT assays and IFN treatment. HeLA and HT1080 (human fibrosarcoma) cells were cultured as monolayers in Dulbecco's modified Eagle medium supplemented with 10% foetal calf serum. DNA transfections and CAT assays were performed as previously described (6). Stable transfectants were generated by co-transfection of pTKneo and subsequent selection in medium containing 400 μg/ml of G418. A highly purified mixture of human α-IFNs was provided by Wellcome Research Laboratories, Beckenham, UK (Wellferon, 1.5 x 10^8 IU/mg protein). Recombinant human γ-IFN (4 x 10^7 IU/mg protein) was supplied by Dr G. Adolf, Ernst Boehringer Institut für Arzneimittelforschung, Vienna, Austria. The IFNs were added to a final concentration of 1000 IU/ml.

Plasmids. The IFN-inducible CAT constructs 203.2.14, 474.1.1 and pPAWcatb′ have been described previously (6), as have plasmids pCe (Ad5 E1A), JF12 (Ad5 E1A 12S alone), JN20 (Ad5 E1A 13S alone), G5/3 (CR1 deletion) and G3/2 (CR2 deletion), which were kindly provided by Dr N. Jones, ICRF (28).

RNA isolation and analysis. Cytoplasmic RNA was prepared from monolayer cells by NP40 lysis and phenol-chloroform extraction and analysed by northern transfer as described previously (6). RNase protection analysis (8) was performed using probes yielding protected fragments of 190 and 270 bp for 6-16 and β-IFN, and 160 and 130 bp for 9-27 and γ-actin respectively. Probes were labelled with [^{32}P]UTP (>400Ci/mmol, Amersham PB.10163) to an activity of 2 x 10^5 cpm of each probe was used to protect 10 μg of cytoplasmic RNA in each assay.

Immuneprecipitation analysis. Monolayer cells (9 cm dish) were incubated for 4 hr in 3 ml of methionine-free medium containing 200 μCi [^{35}S]methionine (>1000Ci/mmol, Amersham SJ1015). Labelled cells were harvested, washed in PBS and lysed in RIPA buffer (Harlow & Lane, 1988). Lysates were pre-cleared with protein A (crude cell suspension, Sigma). After incubation for 1 hr at 4°C with either control serum or hybridoma supernatants of M73 or M2 (Harlow *et al* 1985) protein A (50 μl) was added and incubation continued on a rotator for a further hour at 4°C. Protein complexed with

protein A was pelleted, washed three times with lysis buffer, resuspended in 20 μl of sample buffer, boiled for 2 min and analysed by 8% SDS-PAGE. Gels were fixed, fluorographed with Amplify (Amersham) according to the manufacturer's instructions, and subjected to autoradiography.

Whole cell protein extracts and band shift analysis. Whole cell protein extracts from cells growing as monolayers were prepared as described (21). Protein concentrations were normalized using the BioRad protein assay as per the manufacturer's instructions. Extracts containing 10 μg of protein were incubated with a ^{32}P-labelled 39 bp oligonucleotide TTTACAAACAGCAGGAAATAGAAACTTAAGAGAAATACA corresponding to the 9-27 ISRE (underlined) and flanking sequences (21). DNA-protein complexes were resolved in 6% polyacrylamide gels and visualized by autoradiography. When assaying for the Eα and Eγ subunits of factor E the individual extracts were mixed and incubated for 10 min at room temperature prior to addition of probe. Treatment of extracts with N-ethyl maleimide (NEM) to inactivate Eγ was as described (22).

IFN receptor assays. Interferon binding assays were as described previously (4, 8). Briefly, subconfluent cell monolayers (2 x 10^6 for γ-IFN and 4 x 10^5 for α-IFN) were incubated with ^{125}I-labelled IFN (specific activity of 90 μCi/μg for α-IFN and 180 μCi/μg for γ-IFN) for 90 min on

ice in the presence or absence of a 1000-fold excess of unlabelled competitor IFN. The cells were washed, detached by pipetting in cold binding buffer and the amount of bound radioactivity determined.

Antiviral assays. Cells seeded in 96-well microtiter plates at 5 x 10^4 per well were incubated for 8 hr at 37°C, treated with serial dilutions of α- and γ-IFNs for 18 hr and challenged with 10 pfu/cell of SFV. 50% protection from the cytopathic effects of SFV was scored after a further 24-48 hr.

RESULTS

E1A expression inhibits the response to α- and γ-IFNs in transient transfection assays. Chloramphenicol acetyltransferase (CAT) gene constructs inducible by IFN were co-transfected at a molar ratio of 1:1 into HeLa cells with plasmids designed to express either 12S or 13S E1A mRNAs. Lysates prepared from the transfected cells, with or without IFN treatment, were assayed for CAT activity (Table 3).

Table 3. E1A inhibits α- and γ-IFN inducible expression from CAT constructs in transient co-transfection assays.

A

IFN-inducible component of CAT construct	Fold induction of CAT activity with α- or γ-IFN on co-transfection with					
	control plasmid		12S E1A		13S E1A	
	α	γ	α	γ	α	γ
6-16 gene promoter/enhancer	100.1	8.1	31.2	1.3	37.0	1.4
9-27 gene promoter/enhancer	12.0	7.2	2.3	1.1	ND[a]	ND
6-16 gene ISRE (with tk promoter)	17.5	7.6	2.6	1.3	4.7	1.9

[a]. Not determined

B

CAT expression construct	Relative CAT activity on co-transfection with		
	control plasmid	12S E1A	13S E1A
pBLCAT2	1.0	0.9	3.4
pRSVCAT	1.0	0.8	ND[a]

[a]. Not determined

Co-transfection with the 12S E1A expression construct inhibited the response to α- or γ-IFNs of all of the IFN-inducible constructs tested (Table 3A). This inhibition does not appear to reflect an effect on transcription mediated through a promoter element independent of the ISRE. For each of the constructs, the ISRE is adjacent to very different promoter elements and in one of the constructs the ISRE is 5′ of the herpes simplex virus thymidine kinase (tk) promoter, which is itself insensitive to inhibition by 12S E1A (Table 3B). In addition, high level constitutive CAT expression from a construct under the control of the Rous sarcoma virus long terminal repeat was not inhibited by co-expression of 12S E1A (Table 3B, cf. ref 29), arguing against any general effect on transcription in these cells. A 12S E1A gene product, therefore, appears capable of inhibiting the response to α- or γ-IFNs. Increasing the molar excess of the E1A-expressing plasmid co-transfected with the IFN-inducible constructs increased the level of inhibition (not shown). An inhibitory effect on IFN-inducible expression from the CAT constructs was also observed on co-expression of the 13S E1A construct (Table 3A). In the case of the tk promoter-based ISRE construct (Table 3A) this inhibitory effect was superimposed upon a stimulatory effect on basal transcription (Table 3B). A similar stimulatory effect on the tk promoter has been reported.

Isolation of a human cell line stably expressing E1A products. Human HT1080 cells were stably transfected with constructs (Methods) expressing 12S E1A or 12S plus 13S E1A. Clones were examined for changes in morphology, and for expression of E1A polypeptides and the α- and γ-IFN receptors. Typical results for a clone expressing the 12S products (HT12S1) are presented (Fig 5). The clones show a morphology distinct from that of untransfected cells, growing as very flat, epithelial-like cells (Fig 5A, lower panel). To assay for the expression of E1A, each clone was labelled with [^{35}S]methionine and immune precipitations were performed with monoclonal antibodies specific for the 12S and 13S (M73), or uniquely for the 13S (M2), polypeptide products. The HT12S1 cells expressed two major polypeptides which were precipitated by M73 but not by M2 (Fig 5B). The same two bands, thought to correspond to different phosphorylated forms of the 12S polypeptide, are obtained on translation of the 12S mRNA in a rabbit reticulocyte lysate (Fig 5B).

It was of obvious importance to determine whether expression of E1A affects the cell surface receptors for α- or γ-IFNs. Receptor assays were therefore performed using ^{125}I-labelled IFNs. Scatchard analysis showed that neither the binding affinity nor the cell surface numbers of the receptors were significantly affected by expression of 12S E1A (Fig 5C).

Stable expression of E1A inhibits the cellular response to IFNs and the production of β-IFN in response to dsRNA. HT12S1 and parental HT1080 cells were treated with α- or γ-IFNs for various times and the steady-state levels of 6-16 and 9-27 mRNAs assayed by RNase protection (Fig 6A). The level of IFN-induced mRNA for 6-16, a gene predominantly inducible by α-IFN, was reduced dramatically in the HT12S1 cell line. The accumulation of 9-27 mRNA in response to α- and γ-IFNs was similarly inhibited. Longer exposure of the autoradiographs indicated that although a response to α-IFN was detachable in HT12S1, the response to γ-IFN appeared to be totally ablated (not shown). A similar inhibition of the response to α- and γ-IFNs was observed on analysis of 1-8 and HLA class I mRNAs by northern transfer (not shown). For all of these genes the response to α- and γ-IFNs is through related ISREs.

Induction of the HLA class II genes by γ-IFN depends on element(s) distinct from the type of ISRE present in the genes described above (eg ref 30). Accumulation of HLA class II DRα mRNA in response to γ-IFN was, however, similarly inhibited in the HT12S1 cells (not shown). In addition, induction of β-IFN mRNA in response to dsRNA is inhibited in these cells (Fig 6B). Whether or not common factors are involved, clearly more than one pathway is affected.

The effect of the 12S E1A products on the ability of cells

to mount an antiviral response following IFN treatment was addressed by determining the levels of IFN required to confer 50% protection from the cytopathic effects of Semliki Forest Virus (SFV) in HT1080 and HT12S1 cells. Parental HT1080 cells showed a 50% inhibition of the cytopathic effect at IFN concentrations of 1 U/ml (α-IFN) and 50-100 U/ml (γ-IFN). Concentrations of up to 1000 U/ml of α-IFN and up to 10000 U/ml γ-IFN were insufficient to prevent the cytopathic effects of SFV in HT12S1 cells.

IFN-induced DNA-binding factor activity is reduced in cells expressing E1A. Whole cell extracts were prepared from HT1080 and HT12S1 cells with or without a 4 hr treatment with α-IFN. Band shift analysis was performed using a 39 bp oligonucleotide containing the 14 bp 9-27 ISRE (Methods). Two distinct IFN-dependent complexes were observed (Fig 8A, lane 2). The slower migrating complex E (or ISGF3, ref 31) has previously been shown to be rapidly induced by α-IFN without any requirement for protein synthesis, whereas induction of the faster migrating complex, M (or ISGF2, ref 31), occurs more slowly and requires protein synthesis. A considerable body of evidence supports positive regulatory activities for both of these factors. The levels of E and M observed in response to α-IFN were much reduced in the HT12S1 cell extracts (Fig 7A, lanes 2 & 4).

Treatment of HT1080 cells with γ-IFN induces the binding of

a factor, termed G, which migrates at a similar rate to M, to which it may be highly related. The level of G was also greatly reduced in extracts from HT12S1 cells (not shown).

Active E requires at least two subunits, Eα and Eγ. Eα is activated on treatment with α-IFN. Eγ is constitutive, can be further induced by γ-IFN, but is inactivated by treatment with N-ethyl maleimide (NEM) (22). Extracts from α-IFN-treated cells after exposure to NEM contain only Eα, those from γ-IFN-treated cells contain only Eγ; combination of the two extracts yields functional E. Extracts can, therefore, be assayed for either subunit by complementation with the other (22). Assays of this type indicate that extracts from α-IFN-treated HT12S1 cells have Eα but lack, or have very much reduced, Eγ (Fig 7B). Expression of the 12S E1A appears to inhibit either the synthesis or the activation of Eγ. Consistent with this, extracts from HT12S1 cells were without effect on preformed E, or on its formation from pre-existing Eα and Eγ subunits (not shown).

Sequences within conserved region 1 of E1A are required for inhibition of the IFN response. The IFN response was inhibited in transient co-transfection assays with plasmids expressing either 12S or 13S E1A (Table 3A). Conserved region (CR) 3, which is unique to the 13S product, must, therefore, be dispensable for this function. To determine whether there is a specific requirement for either of the remaining two conserved regions, transient and stable

transfections were carried out with plasmids G5/3 and G3/2 carrying deletions in CR1 and CR2, respectively (28). In transient transfection assays G3/2 (CR2-ve) gave a wild-type repression of IFN-induced CAT activity, whereas G5/3 (CR1-ve) did not (Fig 8A). In stable transformants expression of 6-16 and 9-27 mRNAs in response to α- and γ-IFNs was normal for cells transfected with the CR1-negative G5/3 construct (Fig 8B), despite the fact that they were expressing levels of the CR1-deleted E1A polypeptides comparable to those observed for cells stably transfected with wild-type E1A constructs (not shown). Sequences within CR1 are, therefore, required for the inhibitory effect.

Simultaneous expression of 12S and 13S E1A products is inhibitory. The E1A gene products are capable of transactivation or repression of transcription and it is not clear a *priori* which activity will be dominant in a given situation. Both 12S and 13S products are expressed from the E1A gene on adenovirus infection. It was therefore of interest to determine the effect of expression of both products of the E1A gene in stable transfectants. In cells stably expressing both 12S and 13S mRNAs and E1A proteins (not shown), the expression of 6-16 and 9-27 mRNAs in response to α- and γ-IFNs was inhibited (Fig 9A) and the levels of factors E and M were reduced (Fig 9B). The antiviral state assayed with SFV was much reduced in these cells compared with the parental HT1080 - no protection was

observed with 1000 U/ml of α-IFN or 10000 U/ml of γ-IFN. Consistent with this and the original results of Anderson & Fennie (27), infection of HT1080 cells with wild-type adenovirus reduced the IFN-inducibility of the 6-16 gene and of factors E and M. Such reductions were not observed in cells infected with Ad5 dl312, which lacks the E1A gene (not shown).

Use of cell line in the assay of the invention. The cell lines described above, expressing an IFN-response inhibitory amount of the E1A protein or inhibitory portions thereof (for example the 12S or 13S products), are exposed to a test compound as in Examples 1-5 above to determine whether the inhibition of the IFN-inducible response is reduced.

EXAMPLE 7

A pJ7Ω vector (35) was used as the basis of a construct expressing the HBV TP region (see Example 1 above) under the control of a cytomegalovirus promoter, SCMV IE94.

The hepatoma cell line Hep G2 (36), in which expression of endogenous Class I HLA genes is strongly induced by IFN-α and IFN-γ, was cotransfected with the pJ7Ω-TP construct and pSV2 puromycin, a vector containing a selectable marker gene. Transfected clones were selected with puromycin. Individual clones were assayed for inhibition of the

induction of Class I mRNA and protein expression as above. Cells that failed to respond in these assays were checked for failure of IFN-α and IFN-γ to induce other genes.

The cells are used by introducing test compounds, perhaps of low molecular weight, and assaying for relief of the inhibition of Class I expression as above. The mode of introduction will vary with the test compound.

Class I HLA expression was determined using a northern assay for mRNA induction with a specific Class I probe. To check protein production, a suitable MAb was used, followed by a fluoroscein-labelled second MAb, with fluorescent cells being assayed by FACS (fluorescence-activated cell sorting).

EXAMPLE 8

Any one of the cell lines discussed above is used in an assay according to the invention by exposing the cell line to 500 international units of IFN-α for 24 hours, with or without the candidate antiviral compound, and determining whether the response to the IFN-α is inhibited or not.

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CLAIMS

1. A method of determining whether a test compound is potentially useful for administration to a mammal in or as antiviral therapy, the method comprising the steps of (i) exposing a biological responsive system, which responds to a stimulating substance to cause cytokine expression or cytokine utilisation to increase in the system, to the test compound and to the said stimulating substance, the said system comprising a means for expressing a reporter compound under control of a regulatory region inducible directly or indirectly by the said substance, the said system having a cytokine-response-inhibitory intracellular amount of an inhibitory substance which inhibits the response to the said stimulating substance, and (ii) determining whether the production or fate of the reporter compound is altered according to whether the said test compound is present.

2. A method according to Claim 1 wherein the reporter compound is cytotoxic and its expression is indicated by the death of the cell culture.

3. A method according to Claim 2 wherein the cytotoxic reporter compound is a guanine phosphoribosyl transferase (gpt) and the cells are maintained in a medium containing 6-thioguanine.

4. A method according to Claim 1 wherein the inhibitory substance is HBV polymerase or a cytokine-response-inhibitory portion or analogue thereof (the polymerase or portion or analogue thereof being termed "POL" hereinafter).

5. A method according to Claim 4 wherein the POL is substantially the same as the amino terminal domain of HBV polymerase.

6. A method according to Claim 4 wherein the cells are 2fTGH cells, transfected with means for expressing the inhibitory substance.

7. A method according to Claim 1 wherein the cells are hepatic cells.

8. A method according to Claim 7 wherein the cells are Hep G2 cells, transfected with means for expressing the inhibitory substance.

9. A method according to Claim 1 wherein the inhibitory substance is the E1A protein of adenovirus or an inhibitory portion or analogue thereof or the EBNA 2 protein of Epstein-Barr virus or an inhibitory portion or analogue thereof.

10. A method according to Claim 1 wherein the said stimulating substance is a cytokine.

11. A method according to Claim 10 wherein the cytokine is an interferon.

12. A method according to Claim 11 wherein the cytokine is alpha interferon.

13. A method of determining whether a compound inhibits hepatitis B virus reverse transcriptase (HBVRT) activity comprising (i) providing a system in which HBV POL is expressed, (ii) exposing the system to the test compound and (iii) determining whether the said activity is inhibited.

14. A compound identified as useful by a method according to Claim 1 or 13.

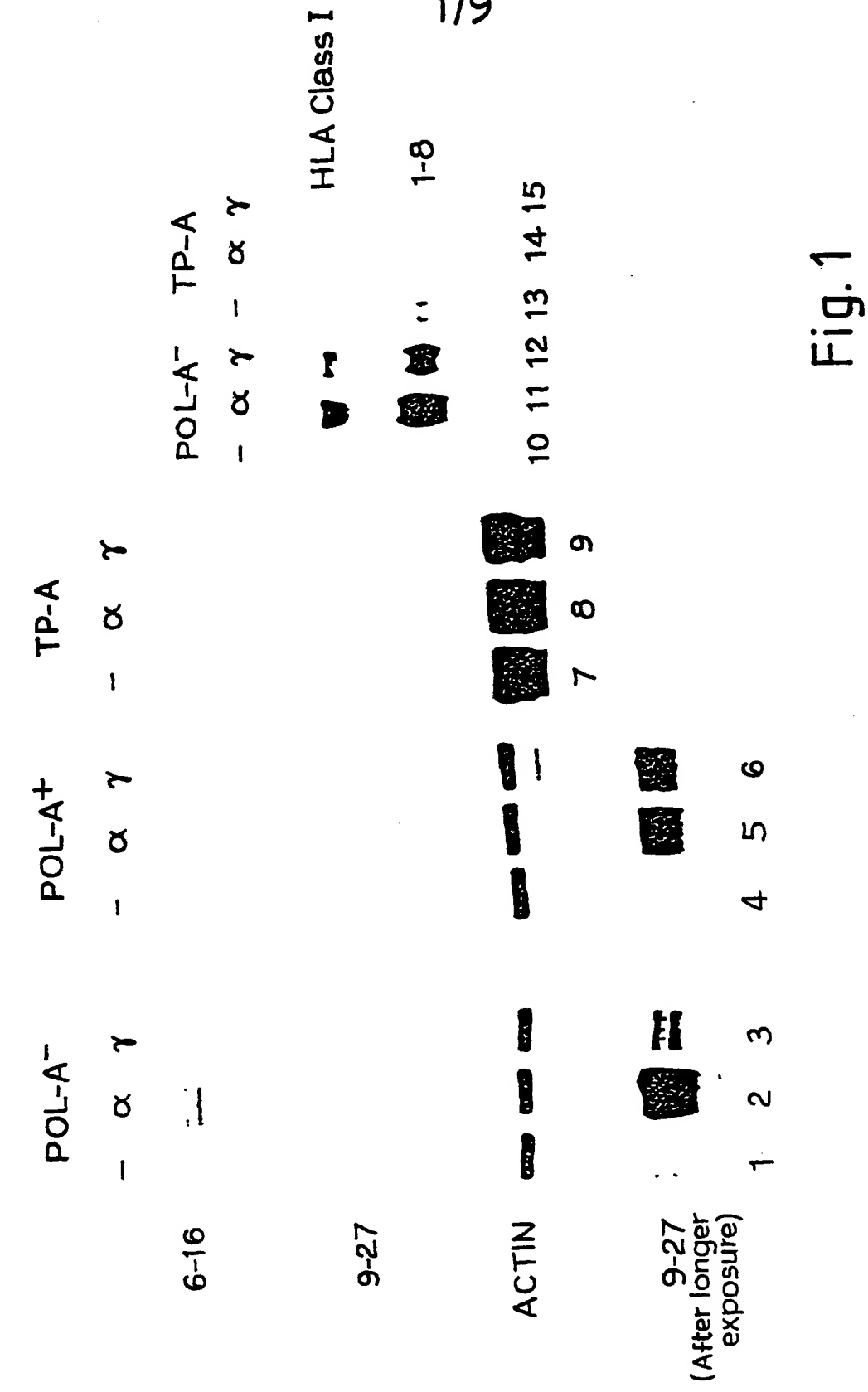

POL-A⁻ TP-A
- α γ - α γ
6-16
POL-A⁻ POL-A⁺ TP-A
- α γ - α γ - α γ
9-27
ACTIN
9-27
(After longer exposure)
1 2 3 4 5 6 7 8 9
POL-A⁻ TP-A
- α γ - α γ
HLA Class I
1-8
10 11 12 13 14 15

Fig. 1

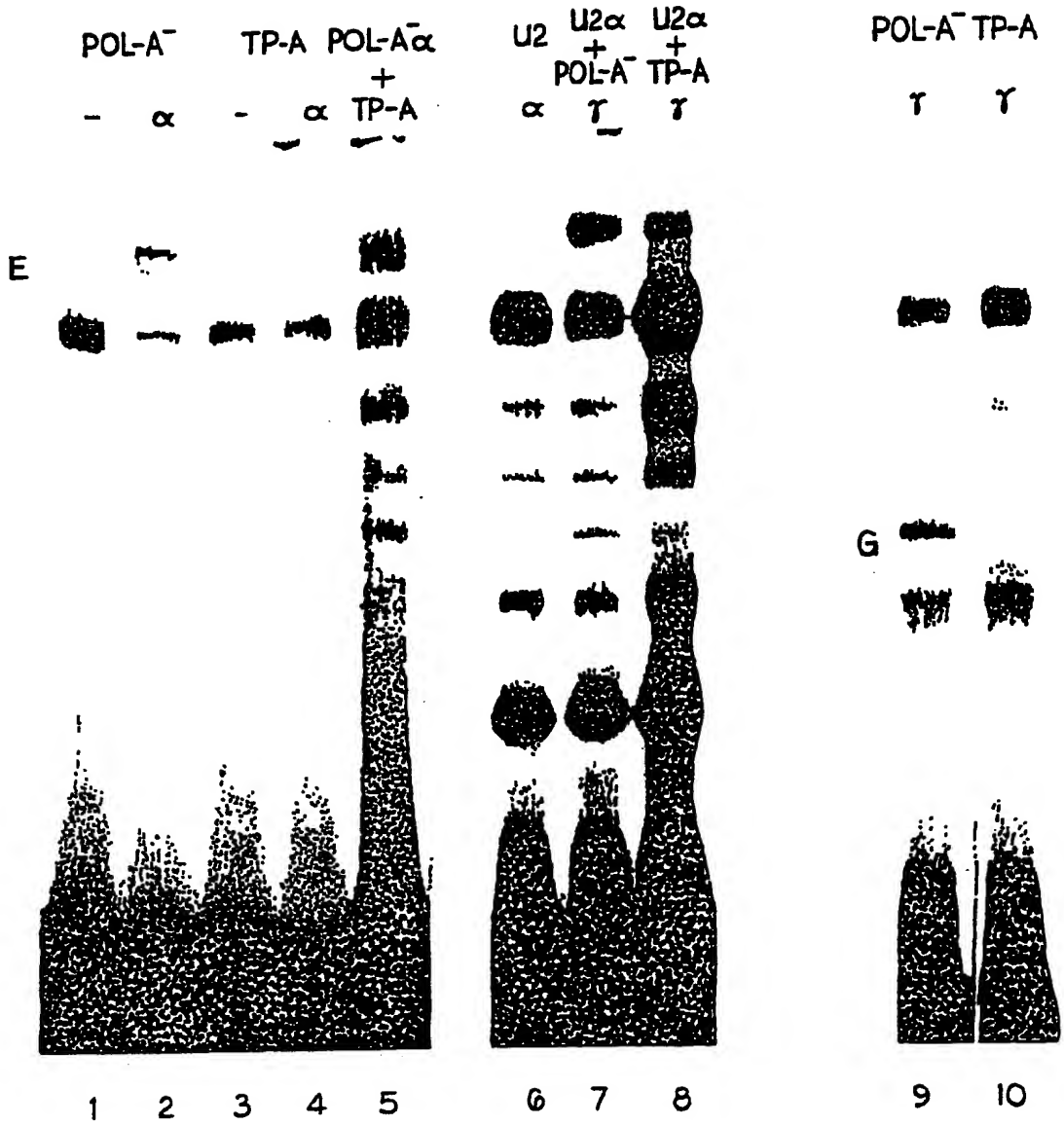
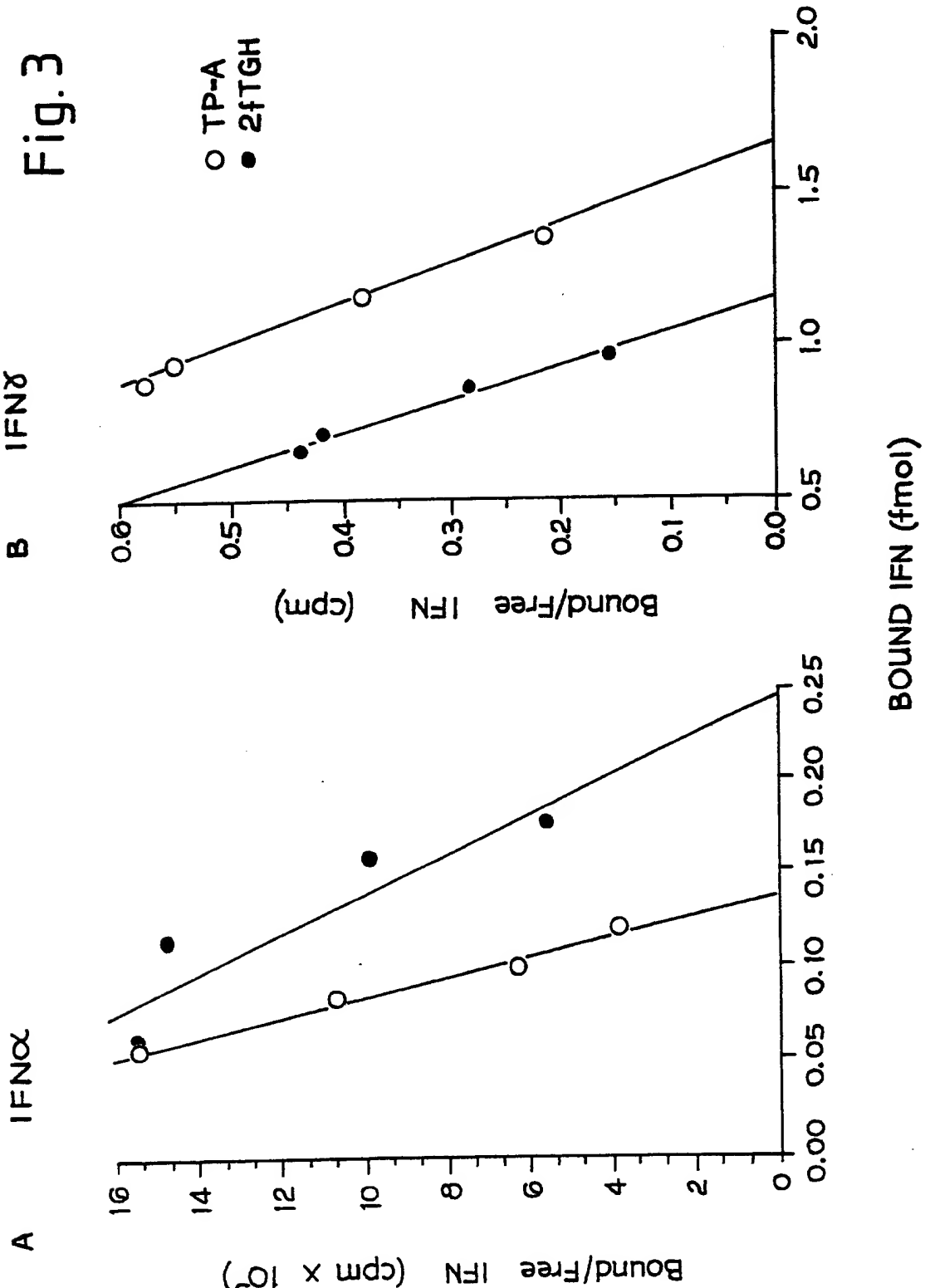

POL-A⁻
TP-A
POL-A⁻α + TP-A
U2
U2α + POL-A⁻
U2α + TP-A
POL-A⁻ TP-A
- α - α
α γ γ γ
E
G
1 2 3 4 5 6 7 8 9 10

Fig. 2

Fig. 3


A
IFNα
Bound/Free IFN (cpm x 10^3)
16
14
12
10
8
6
4
2
0
0.00
0.05
0.10
0.15
0.20
0.25
B
IFNδ
Bound/Free IFN (cpm)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0.5
1.0
1.5
2.0
BOUND IFN (fmol)
○ TP-A
● 2fTGH

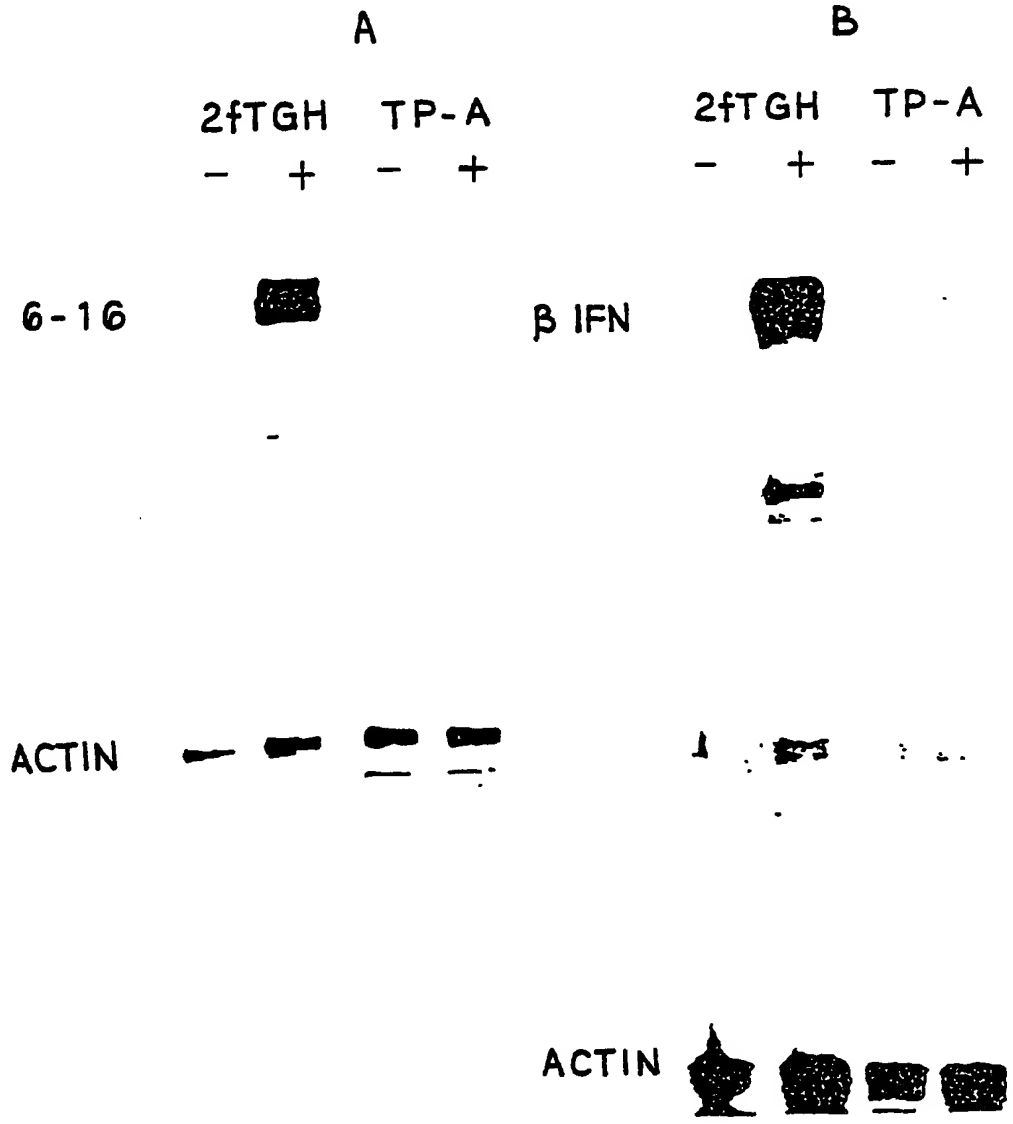


Fig. 4

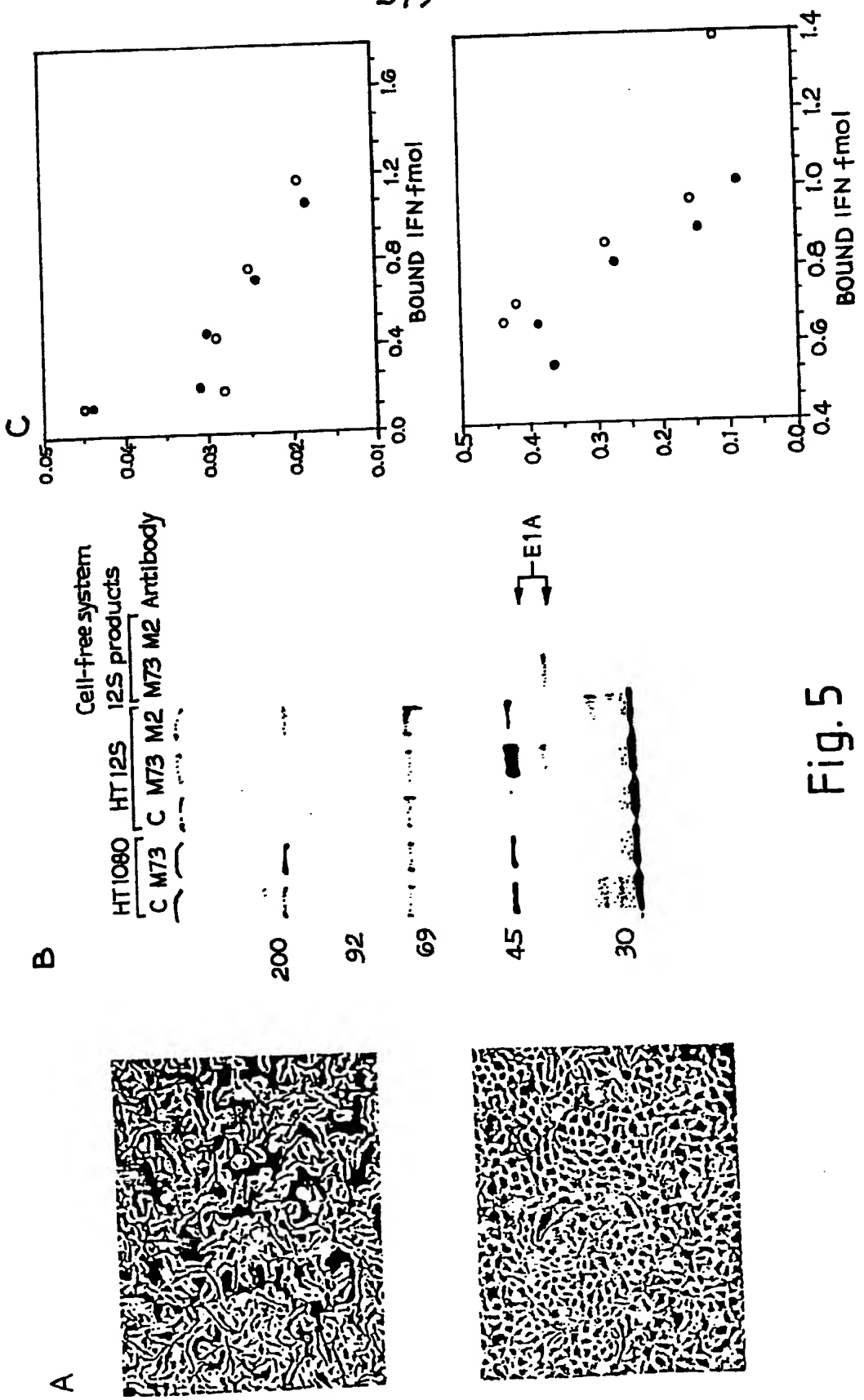
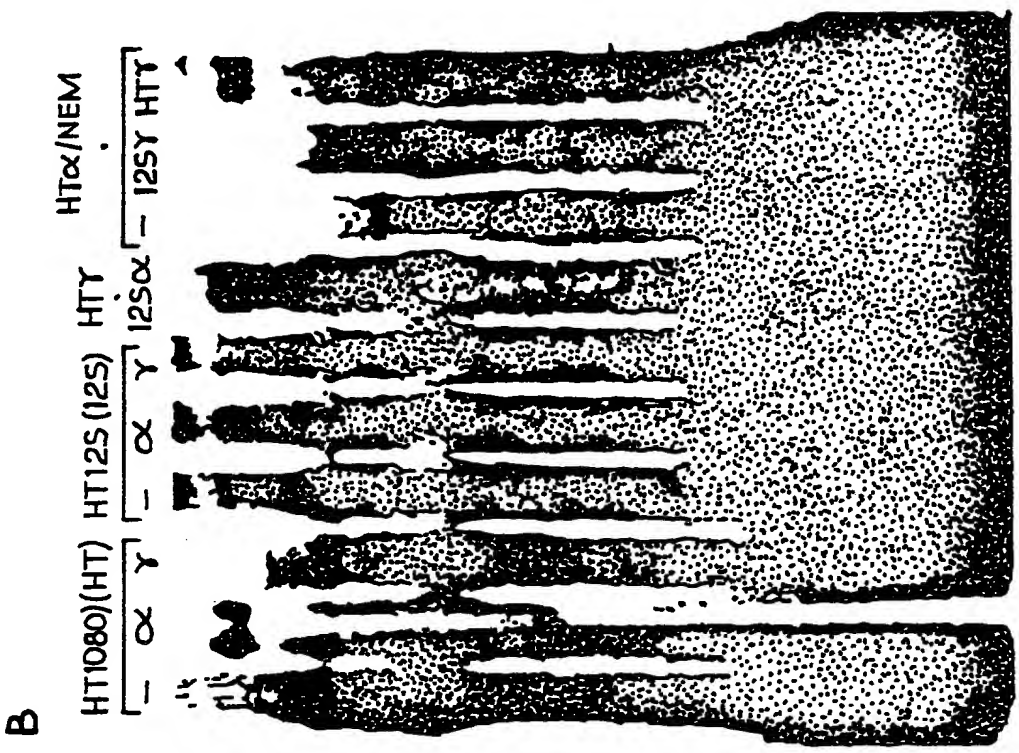

A
B
Cell-free system
12S products
HT1080
HT12S
C M73
C M73 M2
M73 M2 Antibody
200
92
69
45
30
E1A
C
BOUND IFN-fmol
BOUND IFN-fmol

Fig. 5

A
HT1080
α
Y
- 4 8 18 24
HT12S
α
Y
- 4 8 18 24
6-16
9-27
Actin
B
dsRNA
HT1080 HT12S
- + - +
βIFN
Actin

Fig. 6


B
E
HTα/NEM
HTγ 12Sα — 12Sγ HT
HT12S(12S) — α γ
HT1080)(HT) — α γ

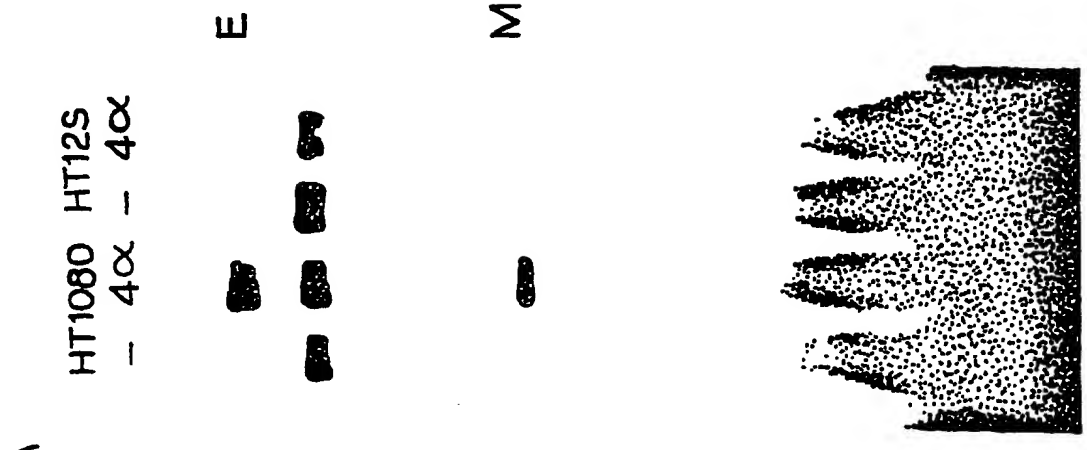

A
E
M
HT1080 HT12S
— 4α — 4α

Fig. 7


B
HT1080
HT12S
HTG5/3
− α γ
6-16
9-27
Actin

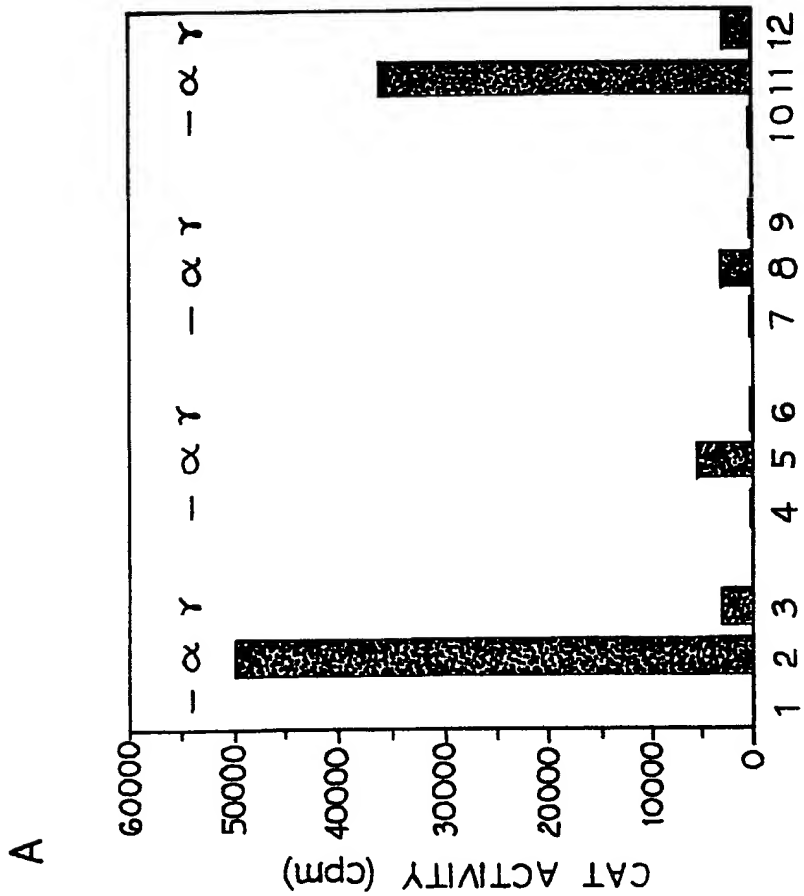

A
CAT ACTIVITY (cpm)
60000
50000
40000
30000
20000
10000
0
1 2 3 4 5 6 7 8 9 10 11 12
− α γ

Fig.8

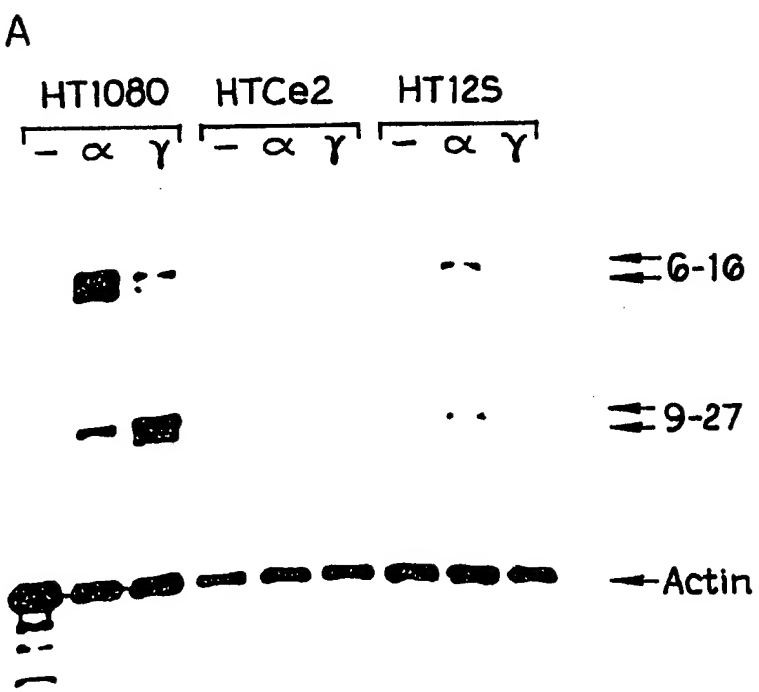


Fig. 9

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 92/00273

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12Q1/18

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12Q ; G01N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
Y	DE,A,2 353 239 (G.D. SEARLE AND COMPANY) 9 May 1974 see the whole document	1-14
Y	JOURNAL OF INTERFERON RESEARCH vol. 10, no. SUP1, 1 November 1990, CHICAGO IL USA page 15; G.R. FOSTER ET AL: 'Hepatitis b pol gene product inhibits the cellular response to interferon.' see whole abstract.	1-14
A	JOURNAL OF HEPATHOLOGY vol. 11, no. SUPP, 1 November 1990, AMSTERDAM NL page 23; G.R. FOSTER ET AL.: 'Hepatitis b pol gene product inhibits the cellular response to type 1 interferon.' see whole abstract.	1-14

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
25 MAY 1992	11. 06. 92
International Searching Authority EUROPEAN PATENT OFFICE	**Signature of Authorized Officer** VAN BOHEMEN C.G.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
T	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA. vol. 88, no. 7, 1 April 1991, WASHINGTON DC USA pages 2888 - 2892; G.R. FOSTER ET AL.: 'Exptression of the terminal protein region of the hepatitis b virus inhibits cellular response to interferons alpha and gamma and double-stranded rna.' see the whole document ---	1-14
T	JOURNAL OF INTERFERON RESEARCH vol. 11, no. SUP1, 1 November 1991, CHICAGO IL USA page 45; I.M. KERR ET AL.: 'Interferon action and its inhibition.' see whole abstract. ---	1-14

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9200273 SA 56336

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 25/05/92

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE-A-2353239	09-05-74	GB-A- 1443425	21-07-76
		AU-A- 6175573	24-04-75
		CA-A- 1012044	14-06-77
		FR-A- 2226073	08-11-74
		JP-A- 49134398	24-12-74